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Immunology

Chromosomal position of rearranging gene segments influences allelic exclusion in transgenic mice

(immunoglobulin gene/rearrangements/B lymphocytes)

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ABSTRACT **Formation of a complete immunoglobulin heavy-chain transcription unit involves the ordered rearrangement of variable (V), diversity (D), and joining (J) region gene segments. In antibody-producing cells, this process is regulated such that only one of two antibody genes is expressed. Experiments with transgenic mice suggest that this mechanism, known as allelic exclusion, is mediated through the membrane-bound form of the immunoglobulin heavy chain. However, in all transgenic lines produced to date exclusion of the endogenous genes by the transgene is incomplete. To characterize the molecular basis for this escape from regulation, we have examined the rearrangements of endogenous immunoglobulin heavy-chain genes. We find that a transgene that encodes the membrane-bound form of human IgM efficiently inhibits rearrangements of endogenous gene segments located at the 5′ end of the heavy-chain locus. However, recombining elements found at the 3′ end of the locus escape and continue to undergo recombination. A transgene that encodes the secreted form of the same immunoglobulin protein has no effect on recombination, regardless of position of the recombining segment in the chromosome. These results have important implications for our understanding of the control of allelic exclusion.**

The antibody genes of B lymphocytes are assembled through a series of genomic rearrangements at the heavy- and light-chain loci (for review, see ref. 1). These events are ordered in that combination of diversity (D) and joining (J) segments precedes variable (V)-to-DJ recombination, and rearrangement of the light chain follows the heavy chain (2). A second level of regulation, referred to as allelic exclusion, insures that only one specific immunoglobulin is produced in any B cell.

There is persuasive evidence that recombination is initiated at both sets of parental loci but is inhibited when one of the two alleles achieves a productive rearrangement (for review, see ref. 2). In addition, experiments with transgenic mice have established that feedback inhibition is mediated only by the membrane-associated form of the immunoglobulin heavy chain (3–5). In these experiments, rearranged human and mouse immunoglobulin transgenes were introduced into the germ line of mice and found to inhibit the expression of endogenous mouse immunoglobulin genes. Furthermore, inhibition of endogenous immunoglobulin gene rearrangements by transgenes has been demonstrated directly in hybridomas, Abelson virus transformants, and lymphomas derived from transgenic mice (5–9). However, none of the immunoglobulin heavy-chain transgenes completely inhibits expression of the endogenous IgM genes (for review, see ref. 10). Although variation between strains is considerable, some leakiness is consistently observed. Failure of the immunoglobulin transgenes to produce complete exclusion could result from (*i*) inadequate expression of the transgene, for any of a number of reasons, including loss of the gene; (*ii*) resistance of specific classes of B cells to the exclusion process (11); (*iii*) trans-recombination between DNA or RNA molecules (12, 13); or (*iv*) inefficient inhibition of recombination of specific gene segments. In this paper, we document that the chromosomal position of rearranging gene segments is a major determinant in the regulation of recombination.

MATERIALS AND METHODS

Nucleic Acid Isolation and Analysis. DNA and RNA from mouse tissues were prepared as described (3). RNase protection assays were done as described by Melton *et al.* (14). Ten micrograms of total bone marrow or fetal liver RNA was hybridized with the appropriate antisense RNA probes overnight at 55°C, followed by RNase digestion. For fetal liver samples, the day in which a vaginal plug was observed was counted as day 0. The hμ-CH4, mμ-CH4, and β2 probes have all been described (3), and the recombination-activating gene 1 (RAG-1) (15) probe was subcloned into Bluescript SK (Stratagene) so that T7 transcription was antisense. β_2-Microglobulin was used as an internal control for the quantity of mRNA. Structures of the RNase protection probes and the expected protected fragments in nucleotides are shown in Fig. 2. The autoradiograms in Fig. 2 represent overnight exposures of 5% acrylamide/7 M urea gels. PCR reactions contained 100 ng of primer, 10 ng of genomic DNA for spleen samples, and 5 ng for B-cell samples and 1 unit of *Taq* polymerase (Amplitaq; Cetus). Hybridization was at 55°C for 2 min, extension at 72°C for 1 min 45 sec, followed by denaturing at 95°C for 30 sec for 30 cycles. One-tenth of the 50-μl reaction was run on a 1.7% agarose gel blotted onto GeneScreen*Plus* and probed under stringent conditions with a 1.1-kilobase (kb) *Apa* I–*Xba* I fragment that covers the J region of mouse IgM. The PCR primers were as follows: 1, J2 GAGAGAATTCGGCTCCCAATGACCCTTTCTG; 2, VQ52 GAGAGAATTCCTGACCATCACCAAGGACAACTCCAAGAG; 3, V7183 GAGAGAATTCAGAGACAATCCCAAGAACACCCTG; 4, VJ558L GAGAGAATTCTCCTCCAGCACAGCCTACATG; 5, 5′D GTCAAGGGATCTACTACTGTG; 6, 5′ IVS GTAAGAATGGCCTCTCCAGGT; and 7, 3′ IVS GACTCAATCACTAAGACAGCT. For cloning PCR, fragments were digested with *Eco*RT and ligated into λZAP (Stratagene). Sequencing was done by the dideoxynucleotide chain-termination method.

Lymphocytes. Spleen cells were obtained from 8- to 12-week-old transgenic TG.SA mice (3), transgenic TG.ST mice (4), and FVB/N wild-type littermate controls. B cells were purified by panning (16). The numbers of B cells in the samples were assayed by staining with Ly-5(B220) monoclonal antibodies (17). The number of B cells did not differ

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Abbreviations: D, diversity; V, variable; J, joining; *RAG-1* and *-2*, recombination-activating gene 1 and 2, respectively.

significantly between transgenic and nontransgenic animals, which agrees with previous results (6).

RESULTS AND DISCUSSION

To test the idea that regulation of allelic exclusion differs for specific immunoglobulin gene segments, we have examined V-to-DJ and D-to-J recombination in transgenic mice that carry human IgM genes. Immunoglobulin V-region segments are clustered in overlapping families spanning hundreds of kilobases (18–21). We used a PCR assay to assess the recombination of specific families of V regions, as well as D and J segments (Fig. 1) (22). The V-region primers are homologous to sequences shared by members of the J558L, VQ52, and V7183 V-region gene families. The D-region primers were selected on the basis of homology to recombination sequences 5′ of all D regions, and the J primers are complementary to regions 3′ of the J2, J3, and J4 regions. Because recombination occurs at all available J regions, a J4-primed amplification results in four sets of specific bands, whereas J2 produces two types of products that correspond to VDJ1 and VDJ2. A pair of oligonucleotides that amplifies a nonrecombining segment in the intervening sequence between J4 and the first constant region was used as a control for the presence of immunoglobulin target sequences. Conditions of the assay were set such that signal intensity is directly proportional to the amount of input target sequence (Fig. 1) (22). PCR experiments were done on DNA from spleen cells and B cells purified from the TG.SA and TG.ST

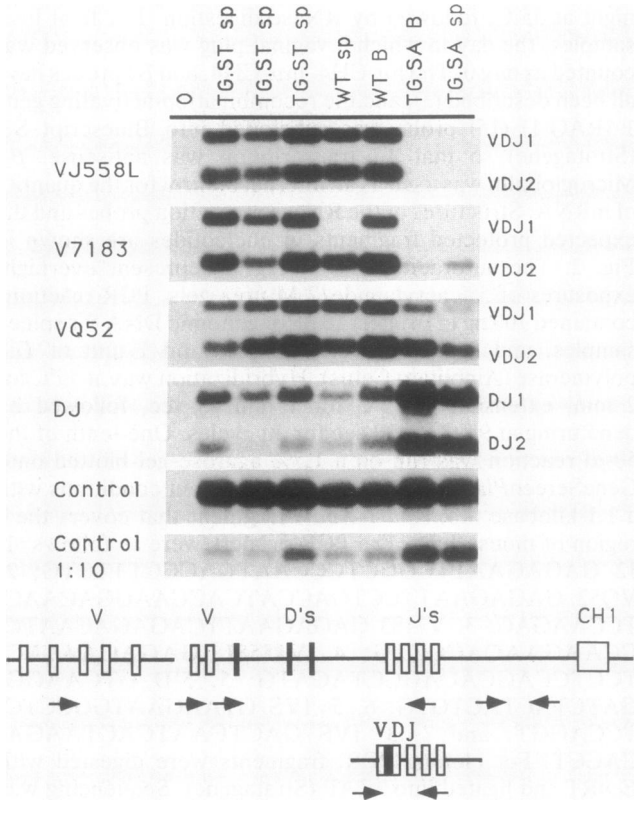

FIG. 1. Immunoglobulin gene rearrangements in transgenic mice assayed by the PCR. Recombination of VJ558L, V7183, VQ52, or D segments with J segments was assayed by PCR amplification and Southern hybridization with an *Apa* I–*Xba* I mouse J-region probe. Control primers are from the J–CH1 intervening sequence. Expected positions of VDJ1, VDJ2, DJ1, and DJ2 recombinations are indicated. 0, no added DNA; sp, spleen; B, B lymphocytes; VJ558L, V558L primer; V7183, V7183 primer; VQ52, VQ52 primer; DJ, D-region primer. CH1, first constant region exon; VDJ, complete heavy-chain recombination.

transgenic strains, as well as wild-type controls. The TG.SA strain carries a human IgM gene modified to direct the synthesis of only the membrane-bound form of the heavy chain (3), whereas the TG.ST strain carries the same gene altered to produce secreted IgM (4). Only the membrane form of the IgM transgene is active in excluding endogenous mouse heavy-chain expression.

In several consecutive experiments with samples from 17 animals, we found that the transgene that encodes membrane-bound IgM dramatically inhibits VJ558L-to-DJ recombination; however, the secreted transgene had no detectable effect (Fig. 1). Inhibition of VJ558L-to-DJ rearrangement by the membrane IgM transgene is consistent with the large decrease in numbers of B cells that express endogenous immunoglobulins in most IgM transgenic strains because VJ558L is the largest of the mouse V-region families and can account for up to 60% of the adult repertoire (23–26). The results for VJ558L also agree with the inhibition of rearrangements detected in Southern blotting experiments (5, 7–9). In contrast to VJ558L, which is the most distal of the V-region families, the more proximal V families—V7183 and VQ52—do undergo rearrangement in B cells from transgenic mice that carry the membrane-bound form of the immunoglobulin transgene. Although there is some inhibition of recombination, especially for V7183 family, there is clearly escape from this type of control in members of both VQ52 and V7183 families (Fig. 1). Because these two V-region gene families account for only a small proportion of the adult repertoire, failure to inhibit these rearrangements in transgenic mice might be expected to have little effect on total surface IgM expression. There was no detectable effect of the membrane or secreted transgenes on recombination of the most proximal recombining elements D and J (Fig. 1). Finally, the same results were also obtained with an independently derived strain of mice that carries a mouse IgM transgene (ref. 27; data not shown).

The differences we observed are not simply from lack of the appropriate target genes because control mouse IgM primers amplified similar amounts of specific sequences from all samples in the same experiment. Furthermore, the samples contained equivalent numbers of B cells, as assayed by surface staining with Ly5 (B220) monoclonal antibodies, and the same results were obtained with purified B cells (Fig. 1). To verify that the amplified DNA represents the results of VDJ recombination, PCR products were cloned and sequenced (Table 1). All but one of the 12 clones sequenced represent independent VDJ joining events. Two of the three VDJ clones amplified from transgenic DNA are out-of-frame VDJ recombinations, and all three of the transgenic clones sequenced contain typical N regions (regions of nucleotide addition or deletion) with nucleotide deletions and additions. We further characterized the recombination machinery in membrane IgM transgenic mice by assaying for expression of RAG-1 and RAG-2 in bone marrow samples (15, 28). β_2-microglobulin was included as an internal control for the amount of RNA in each sample, and the number of B cells was determined by staining with Ly5 (B220) monoclonal antibody. RAG-1 mRNA was readily detected, although the steady-state level is significantly lower in mice that carry the membrane IgM gene (Fig. 2). RAG-2 mRNA levels were lower than RAG-1 mRNA levels and appeared equivalent in transgenic and wild-type mice (data not shown). Despite the lower level of RAG-1 mRNA, all of the machinery required to produce normal recombination is present and active in transgenic B cells (Table 1). The lower level of RAG-1 mRNA may simply reflect accelerated B-cell development induced by the IgM transgene (6).

Our findings are reminiscent of experiments with fetal or neonatal pre-B and B lymphocytes that showed the predominant recombination events in early ontogeny to involve the

Table 1. Sequences of four clones from PCR-amplified spleen DNA obtained from a human membrane IgM transgenic (TG) mouse and a wild-type (WT) littermate

	Regions		
	V7183	D	J
TG	CCGTGTATTACTGTGCAAG	TGGTAACTACT	ACTTTGACTACT
TG	CCTTGTATTACTGTGCAAGAC	ATGGTGACTACG	CTTTGACTACT
WT	CCTTGTATTACTGTGCAAGAC	GACCTTTACT	ACTTTGACTACT
WT	CCATGTATTACTGTGCAAGAC	ATCTGGCCCGATTGTACT	ACTTTGACTACT

more proximal recombining elements (29, 30). One potential explanation for our observations would be that the membrane-bound IgM gene is not expressed in early B cells. Under these circumstances, the normal fetal repertoire would become established, followed by inhibition of rearrangements responsible for producing the adult repertoire. However, lack of expression cannot account for our observations because the human IgM transgene is expressed in large quantities in fetal liver (Fig. 2). On embryonic day 18 when endogenous IgM is just detectable transgenic mRNA is already abundant. In addition, the transgene is expressed in pro-B and pre-B cell lymphomas (6, 31). Another explanation for our results would invoke the existence of another class of B cells present in the fetus that are unable to transduce signal from the membrane-anchored IgM to the nucleus. This explanation would require a class of B cells present during early ontogeny that fails to either produce or use the signal-transduction apparatus. But even this additional class of B cells would not adequately explain the continuing effect of gene-segment position on rearrangement. Although the mechanisms responsible for the skewing of fetal V_H usage has not been elucidated, these observations imply differential

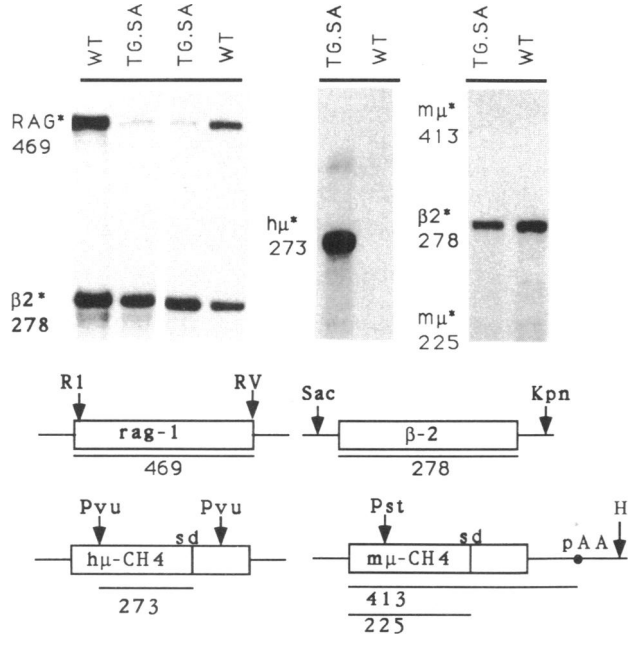

FIG. 2. RNase protection experiments in transgenic mice and their wild-type littermates. (*A*) Expression of RAG-1 and β_2-microglobulin mRNAs in bone marrow samples from two wild-type and two TG.SA transgenic mice. β_2-microglobulin is used as an internal control for the amount of mRNA in the samples (3). (*B*) Expression of membrane form of human IgM, mouse IgM, and β_2-microglobulin in transgenic and wild-type day-18 fetal liver samples. β-2, β_2-microglobulin exon 2; RAG, RAG-1; R1, *Eco*RI site; RV, *Eco*RV site; Pst, *Pst* I site; Sac, *Sac* I site; Kpn, *Kpn* I site; Pvu, *Pvu* I site; Pst, *Pst* I site; H, *Hin*dIII site; sd, splice donor; hμ-CH4, human IgM constant region exon 4; mμ-CH4, mouse IgM constant region exon 4; pAA, polyadenylylation/cleavage site. *, approximate position of the expected protected fragment.

regulation of recombination for proximal recombining segments. Our experiments show that these gene segments fail to respond to the signals generated by membrane IgM.

Substantial experimental evidence supports the idea that recombination is regulated by transcription of specific gene segments (for review, see ref. 1) and that the immunoglobulin enhancer can activate recombination (32). We propose that the effect of chromosomal position on regulation of recombination is a function of the strong transcriptional activators present in the immunoglobulin heavy-chain enhancer. Activation of the enhancer would cause early rearrangement of proximal segments, whereas more distal segments may require expression of additional regulators. In this model, membrane IgM could inhibit rearrangement of distal V_H families by inhibiting the activity of distal regulators. However, the same membrane-anchored heavy chain would have little effect on more proximal recombining elements because continued enhancer activity is required for mature immunoglobulin gene transcription (33). Definitive exclusion of the proximal segments from recombination would require shutting off the recombination machinery, which can occur only after light-chain gene rearrangement. This two-step model for allelic exclusion could also account for the observation that there is normally no exclusion of DJ recombination and that V-region replacement (34, 35), when it occurs, involves proximal V regions.

Serologic and mRNA expression measurements have documented that V_H use is biased in independently derived transgenic strains that carry a mouse IgM gene with a different V region (36, 37). Our experiments confirm these earlier studies and extend them by providing a molecular basis for the skewed repertoire, specific inhibition of recombination in a position-dependent fashion. In the M54 and M95 strains, the transgenic 17.2.25 V region is a member of a proximal V-region family (9), and the product of the transgene was postulated to participate in a selective feedback loop that is, in some way, specific for the proximal transgenic V region. The observation that squewed inhibition of recombination occurs in mice that carry the membrane transgene but not in mice that carry a secreted transgene rules out any selection effect that is either isotype or idiotype specific (Fig. 1) (36, 37). If selection were to account for our findings, the mechanism would have to be an idiotype- and isotype-independent reversal of the normal process that causes high-level representation of distal V regions. Furthermore, the human V region in the transgenes used in our studies is closely related to the J558L family, which is located in the 5′ end of the immunoglobulin locus (18–21). Despite similarity of the human transgene to distal V-region families, we find that it is the proximal V regions that escape allelic exclusion. In conclusion, escape from exclusion of proximal recombining gene segments in transgenic mice is independent of the type of transgenic V region and is likely an important feature of the exclusion mechanism.

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